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Jendis, J ; Strack, B ; Volkmann, S ; Böni, J ; Mölling, K

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# Inhibition of Replication of Fresh HIV Type 1 Patient Isolates by a Polypurine Tract-Specific Self-Complementary Oligodeoxynucleotide

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## ABSTRACT

A previously described self-complementary oligodeoxynucleotide termed triplex-forming oligodeoxynucleotide (TFO A), 54 bases in length, designed against the polypurine tract of HIV-1 RNA, inhibited viral replication at a 1 to 3  $\mu\text{M}$  concentration in acutely infected cells, whereas antisense and scrambled sequence oligodeoxynucleotides were ineffective. Three HIV-1 viral isolates from patients of clinical categories A1, B, and C3 were transmitted to peripheral blood mononuclear cells and tested for production of p24 antigen and syncytium formation in the absence and in the presence of either TFO A or a control oligodeoxynucleotide of randomized sequence. No p24 antigen or syncytia were detected for up to 30 days when TFO A was added to the cells. Viability of the cells was found not to be affected by the drugs compared to controls within 2 weeks. Analysis of viral DNA synthesis by PCR for the LTR and *gag* gene indicated no DNA signal, suggesting that TFO A affects viral replication before formation of a DNA provirus.

Measurements of the stability of TFO A indicate a half-life of about 2 hr. A two-dimensional computer fold analysis of TFO A suggested a self-complementary hairpin-loop configuration with GC-rich stems and single-stranded 5' and 3' ends. Since intracellular triplex formation may not be an efficient process, the observed inhibitory effect may be due to a direct inhibition of the RT and RNase H enzyme activities by the oligodeoxynucleotide. However, a triple-helix effect on the incoming RNA may play a role as well.

## INTRODUCTION

WE HAVE DESCRIBED a culture system based on the acute infection of an HTLV-I-transformed T cell line with the human immunodeficiency virus type 1 (HIV-1) to evaluate the long-term efficacy of antiretroviral treatment based on a triple helix-forming oligodeoxynucleotide.<sup>1</sup> It has been shown that a synthetic, 54-base long oligodeoxynucleotide (ODN), termed triplex-forming oligodeoxynucleotide A (TFO A), could inhibit HIV-1 replication in an acute infection, when virus was added to an uninfected susceptible cell line C81-66/45. This oligodeoxynucleotide comprises 25 bases complementary to the extended polypurine tract (PPT) sequence.<sup>2</sup> A linker consisting of four thymidines connects this part of the molecule to a second, 25-base long sequence that, by virtue of Hoogsteen base

pairing with the preformed RNA-DNA hybrid, is capable of triple-helix formation. The four nucleotides at either terminus and the (T)<sub>4</sub> linker were modified by phosphorothioates. This molecule is capable of exerting antiviral activity based on triple-helix interaction. This is because the heteroduplex forms around a purine-rich target.<sup>3</sup>

The polypurine tract serves an important function in retroviral replication since it provides the primer for the second-strand DNA synthesis. After a full-length minus-strand DNA copy has been synthesized the RNase H activity of the reverse transcriptase (RT) catalyzes the hydrolysis of the viral genomic RNA in the RNA-DNA hybrid.<sup>4</sup> RNA complementary to the PPT is resistant to RNase H cleavage and serves as primer for second-strand DNA synthesis by the DNA-directed DNA polymerase activity of the RT. This step is essential for retroviral

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replication and requires a functional RNase H activity.<sup>5</sup> The PPT is a highly conserved region among retroviruses located adjacent to the unique region at the 3' end (U3) of the viral genome in the coding region of the HIV *nef* gene. A second PPT is located in the coding region of the integrase gene.<sup>6</sup> The PPT can function as a target for triple-helix formation. Formation of Watson–Crick base pairs between the 3' half of TFO A and the RNA would result in an RNA–DNA hybrid that is a substrate for RNase H activity. Using conditions in which a triple helix could form, no RNase H cleavage was detected, indicating the formation of Hoogsteen base pairs between the RNA and the 5' half of TFO A.<sup>1</sup> However, *in vitro* in the presence of higher concentrations of TFO A (>1  $\mu$ M) the RT and the RNase H activity are directly inhibited, as shown by primer-extension experiments and RNase H assays using a random hybrid substrate. In contrast to these *in vitro* experiments the mechanism of TFO A inhibition of retroviral replication in cell culture has not yet been elucidated. The drug is effective at 1  $\mu$ M concentration in the culture medium, a concentration that is *in vitro* at the borderline between a triple-helix effect and a direct enzyme inhibition. Whether a synergy of both effects exists intracellularly is not known.

The TFO A used here contained phosphorothioate modifications at the 5' and 3'-terminal ends and at the (T)<sub>4</sub> linker to make it resistant against exonucleases and suitable for its application in cell culture. The oligodeoxynucleotide is designed to bind to an extended 25-mer purine-rich target sequence that encompasses the contiguous 16-mer PPT, two nonpurine bases, and an additional seven purines. In base triplets containing a Watson–Crick GC base pair, guanine was used as a third strand base, rendering the triple helix much more stable under physiological conditions.<sup>1</sup>

Here we present further evidence for the antiviral efficacy of TFO A and present data that this oligodeoxynucleotide can also inhibit viral replication of primary clinical HIV-1 isolates obtained from three seropositive individuals and transmitted to peripheral blood mononuclear cells (PBMCs) in culture. Furthermore, we show that the TFO A exerts an inhibitory effect before DNA provirus formation.

## MATERIALS AND METHODS

### Oligodeoxynucleotides

Oligodeoxynucleotides (ODNs) were synthesized on an Applied Biosystems (Foster City, CA) 380 B synthesizer using standard phosphoramidate chemistry and were purified as described.<sup>1</sup> Phosphorothioated ODNs were purchased from Biotech (Berlin, Germany). The following ODNs were used: phosphorothioated TFO A (5' *TsTsTsTCTTTGGGGGGTTGGTTGGG**sTsTsTsTCCCTTCCAGTCCCCCTTTTCTsTsTsT*), consisting of a Watson–Crick-forming sequence of 25 nucleotides directed against the extended PPT (underlined), a triple helix-forming sequence of 25 nucleotides (italics), connected by a T linker of 4 nucleotides. The *s* indicates the thioate modifications. A scrambled version of TFO A, used as control, was named TFO SC (5' *TsTsTsGGGGGGTTCTTCTCTTCTCTTsTsTsTsTCGCCCGTCCGTTGCGTTGATTTsTsTsT*). As a further specificity control TFO B (5' *TsGsTsGTGTGTGTG*

*TGTGTGTGTGTGTsTsTsTsTCCCTTCCAGTCCCCCTTTTCTsTsTsT*) was used. Furthermore, a thioated 16-mer PPT antisense ODN designated AS 16 (5' TCCCCCTTTTCTsTsTsT) corresponding to the Watson–Crick base-pairing sequence of the TFO A, and a scrambled derivative of the latter termed KO 16 (5' TCCTCTCTTCTTsCsTsTsT), were tested for antiretroviral activity. Also, the corresponding 16-mer ODNs protected by an amino group at their 3' end were used.

### Assay for inhibition of HIV-1 replication in cell cultures of the T cell line C81-66/45

Phosphorothioate ODNs and ODNs modified by an amino group replacing the 3'-hydroxyl group were tested for antiviral activity in a cellular acute infection assay. In this assay the HTLV-I-transformed T cell line C81-66/45 was used (kindly provided by R. C. Gallo, NCI, NIH, Laboratory of Tumor Cell Biology, Bethesda, MD).<sup>7</sup> To study the effect of the ODNs in cell culture, the following the TFOs were used: TFO A, TFO SC; in addition, thioated 16-mer PPT antisense ODNs designated AS 16, and scrambled derivatives of these ODNs that were either phosphorothioate modified (AS 16 S; KO 16 S) or protected by an amino group at their 3' end (AS 16 A, KO 16 A), were tested for antiretroviral activity. HIV-1 replication was investigated in experiments as described.<sup>1</sup> Briefly,  $2 \times 10^5$  C81-66/45 cells/well of a 48-well plate were infected with HIV-1 strain HTLV-IIIb<sup>8</sup> containing supernatant that had been titrated for determination of infectivity. We used a low multiplicity of infection (MOI 0.05). Viable cell number increased to  $1 \times 10^6$  cells/ml within 4 days of incubation as determined by trypan blue exclusion. Once a week viable cells were counted and split down to  $1 \times 10^6$  cells/ml. HIV-1 infection of C81-66/45 cells is characterized by the appearance of large syncytia in culture that can be detected by microscope. Two hours postinfection the cells were washed and treated with the ODNs at indicated concentrations in culture medium (first spike). After 48 hr the supernatant was removed and fresh medium was added again containing the ODNs at an identical concentration (second spike). After 4 to 5 days medium was replaced by fresh medium without ODNs for the remainder of the cultivation periods. Virus production was monitored at the cellular level by the appearance of syncytia and in the supernatants by p24 antigen-capture assay using the Du Pont HIV-1 p24 core profile ELISA (Du Pont de Nemours GmbH, Bad Nauheim, Germany). At the time points indicated, culture supernatant was removed for p24 antigen analysis and replaced by fresh medium. Supernatants were tested in serial dilutions and p24 values indicated in Figs. 1–3 were derived from the linear range. In these experiments the effect of TFO A in comparison to TFO SC, the pair of PPT antisense ODNs (AS 16 S, AS 16 A), and their control ODNs (KO 16 S, KO 16A) mentioned above, were analyzed over a time period of 17 days.

### Assay for inhibition of replication of fresh HIV-1 patient isolates in cultures of peripheral blood mononuclear cells of healthy Swiss blood donors

The HIV isolates used for these experiments were obtained as supernatant virus from cocultures established from the PBMCs of three HIV-1-seropositive individuals, consisting of

one asymptomatic (A1) and two symptomatic (B and C3), with PBMCs obtained from buffy coats of healthy seronegative Swiss blood donors as recipient cells for the virus. Cocultures were performed according to a method described by Ho *et al.*<sup>9</sup> Supernatants of the cocultures were saved with each medium change, stored at 4°C, and later tested for p24 viral antigen. Nine days postinfection maximum amounts of viral core antigen could be demonstrated in the culture supernatant. These supernatants were then subjected to filtration through a 0.45- $\mu$ m pore size nitrocellulose filter (Sartorius, Goettingen, Germany) to remove all cells and cell debris and then added to freshly prepared PBMCs from another normal seronegative Swiss blood donor to serve as target cells for HIV-1 replication of the new viral isolate. PBMCs ( $1.8 \times 10^6$ ) were cell-free infected with the virus for 2 hr and split into three parts, with and without TFO A treatment as well as scrambled (TFO SC) control added at 1  $\mu$ M concentration in two spikes; see Fig. 3A–C.

#### Detection of viral DNA in peripheral blood mononuclear cell cultures by polymerase chain reaction

Viral DNA in infected PBMCs was detected by a qualitative polymerase chain reaction (PCR) analysis. Total cellular DNA was prepared by detergent lysis and proteinase treatment according to the method described by Higuchi.<sup>10</sup> Amplifications with the DNA equivalent of  $2 \times 10^5$  cells per reaction were carried out in duplicate for 35 cycles using primers HIV-L1 and L3, and HIV-G1 and G2 for the long terminal repeat (LTR) and *gag* regions, respectively.<sup>11</sup> The resulting products were identified by a nonisotopic detection system that is based on solution hybridization of the amplified DNA with two oligodeoxynucleotide probes that are labeled with biotin and digoxigenin. After binding of the hybrid molecules to a solid phase, the digoxigenin tag is detected by an enzyme linked immunosorbent assay.<sup>12</sup> Oligonucleotide probes used for *gag* and LTR sequences were, respectively, HIV-G3-BIO (5' AGTGGATAATCCTGGGATTAATA 3') and HIV-G7-DIG (5' AATAGTAA-GAATGTATAGCCCT 3'), and HIV-L2-BIO (5' GGTCT-GAGGGATCTCTAGTTACCAG 3') and HIV-L7-DIG (5' GCACTCAAGGCAAGCTTTATTGAGGC 3'). This PCR analysis has the ability to detect a single copy of HIV-1 DNA.

#### Analysis of triplex-forming oligodeoxynucleotide stability

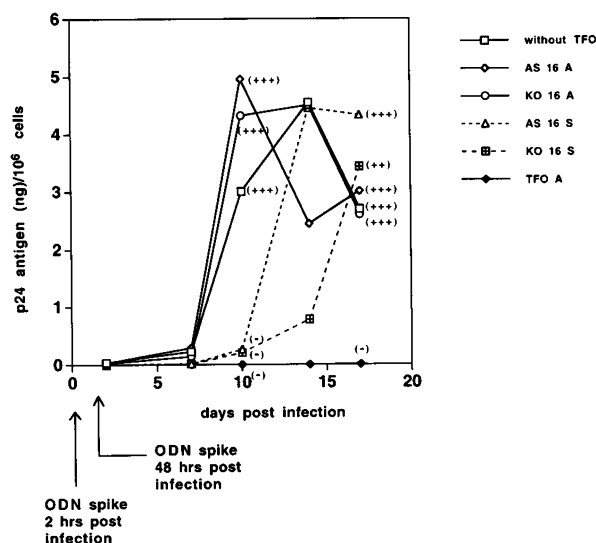
TFO A and TFO SC were 5' end-labeled using [ $\gamma$ -<sup>32</sup>P]ATP in the presence of T4 polynucleotide kinase. The radioactively labeled TFOs were added to 1 ml of C81-66/45 cells ( $2 \times 10^5$  cells/ml of RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum) to a final concentration of 1  $\mu$ M ( $1 \times 10^8$  cpm/pmol). After various times of cell growth 50- $\mu$ l aliquots of the cell suspension were taken and the cells were lysed by addition of 20  $\mu$ l of lysis buffer (400 mM NaCl, 40 mM Tris-HCl [pH 8.0], 100 mM EDTA, and 2% sodium dodecyl sulfate [SDS]). For definition of the zero time point, the 50- $\mu$ l aliquot was taken immediately after the addition of the TFOs to the cells. After phenol extraction an aliquot of the aqueous phase was subjected to electrophoresis using a denaturing 12% polyacrylamide gel containing 7 M urea. The amount of undegraded TFO was quantitated by means of phosphorimag-

ing. The so-called splayed-arm structure consisting of a branch point with adjacent single-stranded regions has been shown to be the substrate of a new type of endonucleolytic activity.<sup>13</sup>

## RESULTS

### Inhibitory effect of oligodeoxynucleotides on HIV-1 infection of T cells

The HIV RNA genome contains two polypurine-rich sequences (PPTs). These PPTs constitute potential target sites for triple-helix formation. Blockage of the biological function of these sequences could also be accomplished by the use of antisense ODNs directed against the PPT. Syncytium formation was determined by microscope and graded and p24 production measured by enzyme-linked immunosorbent assay (ELISA). We first compared the antiviral effect of TFO A with 16-mer antisense ODN (AS 16 A and AS 16 S, 1  $\mu$ M concentration) allowing Watson-Crick base pairing at the PPT. Oligodeoxynucleotides with a randomized sequence were used as control (KO 16 A and KO 16 S). No inhibitory effect of the AS is observed when the kinetics of p24 production are compared to those of the cultures that received the control or no ODN treatment (Fig. 1). In all three cultures p24 productions



**FIG. 1.** Antiviral effects of phosphorothioate-modified TFO A in comparison to its corresponding 16-mer antisense ODN (AS 16) and a scrambled derivative ODN (KO 16) that were either phosphorothioate modified (AS 16 S; KO 16 S) or protected by an amino group at the 3' end (AS 16 A; KO 16 A). Cultures consisted of C81-66/45, an HTLV-I-transformed T cell line newly infected with HIV-1 type IIIB. After 2 hr of incubation with the virus cells were washed and treated with the ODNs (1  $\mu$ M). The second treatment was performed 48 hr later, including a change of the medium (RPMI 1640 plus 20% heat-inactivated fetal bovine serum [GIBCO, Life Technologies, Basel, Switzerland]). As control one culture was tested without added ODN. Supernatants were collected and viral antigen expression determined by p24 antigen capture assay. The amount of p24 (ng) per  $1 \times 10^6$  cells was plotted as a function of time. Syncytium formation was determined by microscope and is graded (–, +/– to ++++).

started at day 7 postinfection, increased subsequently to peak levels, and declined gradually thereafter. Slightly better protection results were obtained with a 16-mer antisense ODN, protected against degradation by three phosphorothioated deoxynucleotides at either end (AS 16 S), and a randomized control (KO 16 S). Treatment of infected cells with these phosphorothioate derivatives prevented syncytium formation for up to 10 days. Figure 1 shows that development of high levels of viral replication in the culture treated with AS 16 S occurred 4 days later as compared to cultures that received either AS 16 A or its control. However, the randomized phosphorothioate control KO 16 S exhibited an even more pronounced delay on viral replication, indicating nonspecific effects. In contrast to that, treatment of cells with TFO A led to a complete inhibition of viral replication as measured by p24 production.

#### *Inhibition of HIV-1 infection by TFO A is dose dependent*

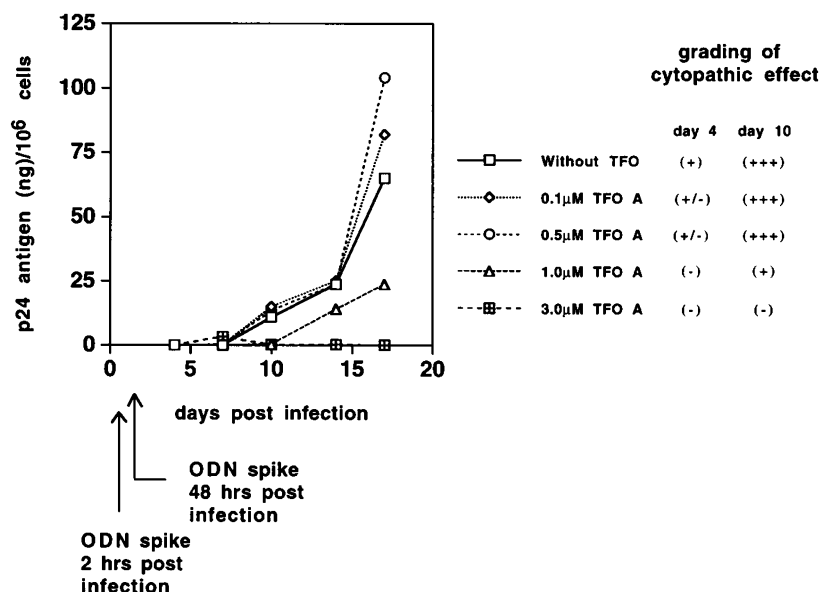
To elucidate further the antiviral effect of the most potent inhibitor, TFO A, a variety of different concentrations of the compound were tested. A dose-response curve for p24 production as a measure of HIV-1 IIIB inhibition is shown (Fig. 2). TFO A was added twice as described above. Protection against HIV-1 cytopathicity is achieved at 1 to 3  $\mu$ M concentrations. Up to 10 days after infection no cytopathic effect is observed in these cultures at the higher TFO A concentration. HIV-1 p24 production in the culture supernatant is inhibited by 64% at 1  $\mu$ M concentration in comparison to the culture without TFO treatment. A beginning cytopathic effect in this culture is noticed only 10 days postinfection. Thus, at 1  $\mu$ M concentration the drug is able to effectively delay virus production and the onset of the cytopathic effects in acutely infected CD4<sup>+</sup> T cells. Higher amounts of p24 antigen production were measured in the supernatants in cultures containing 0.1 and 0.5  $\mu$ M

concentrations of TFO A than in untreated cultures 17 days postviral infection. A complete inhibition is achieved at a 3  $\mu$ M concentration of TFO A, which is higher than previously described and where a complete inhibitory effect was observed with 1  $\mu$ M TFO A.<sup>1</sup>

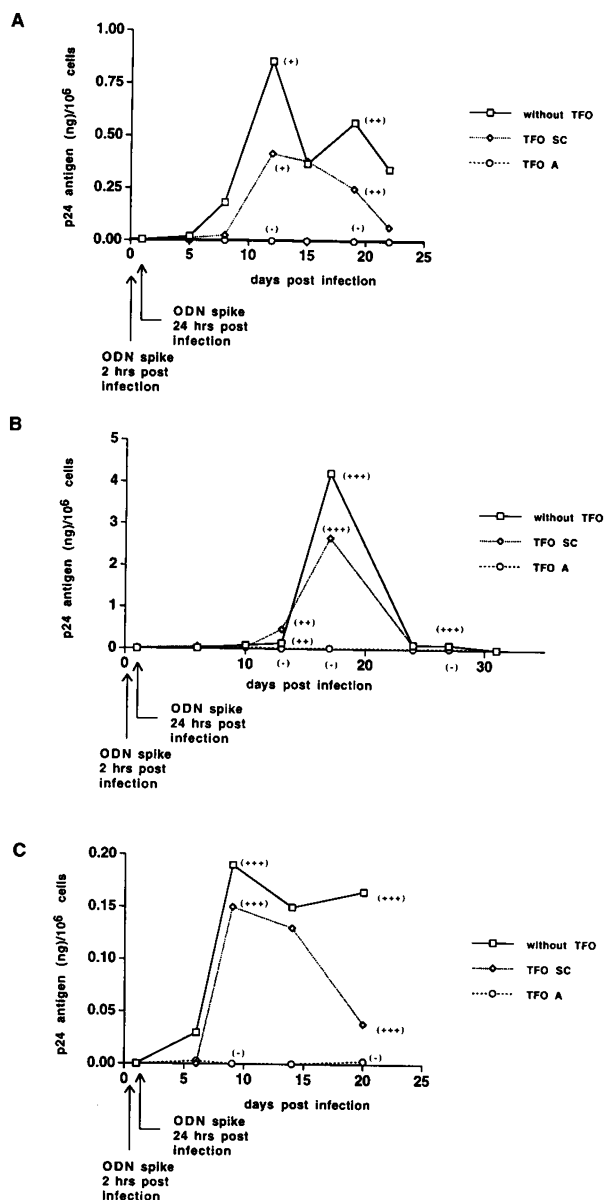
We have investigated antiviral activities of antisense and triplex-forming oligodeoxynucleotides by using acutely infected cells of an established T cell line as assay system. To exclude the possibility that TFO A can inhibit only a single laboratory propagated viral strain, we also used three patient isolates for elucidation of the biological effects of TFO A.

#### *TFO A inhibits the infection of peripheral blood mononuclear cells with clinical HIV-1 isolates*

We used a two-step procedure to obtain and test the HIV isolates. First, the PBMCs of the seropositive individual (asymptomatic of category A1 of HIV infection) were cocultured with PBMCs prepared from a seronegative blood donor at a 1:1 ratio. Filtered supernatants that contained high amounts of p24 antigen were then transmitted to PBMCs obtained from a second seronegative blood donor as target cells for viral replication. This two-step cell-free virus transmission resembles the previously described acutely infected C81-66/45 cell system.<sup>1</sup> Cells were incubated with the virus for 2 hr. Infected cells were then washed and TFOs were added with fresh medium. In contrast to the previously described experiments using the cell line C81-66/45 new medium supplemented with TFOs was added again after 24 hr. TFO A completely blocked viral replication as measured by p24 production (Fig. 3A). Furthermore, no cytopathic effect was detected in this culture. In contrast, p24 production was observed in the culture treated with the random sequence ODN TFO SC and in the control culture that received no treatment. The culture treated with TFO SC produced slightly lower amounts of virus throughout the culture period



**FIG. 2.** Antiviral effects of increasing concentrations of TFO A. TFO A was added twice, at 2 and 48 hr following viral infection, to C81-66/45 T cells at concentrations as indicated. Determination of p24 expression and syncytium formation was performed as described in the caption to Fig. 1.



**FIG. 3.** Antiviral effects of TFO A and TFO SC on fresh HIV patient isolates transmitted to cultures of PBMCs of healthy seronegative Swiss blood donors. HIV isolates were obtained as supernatant virus from cocultures established from PBMCs of an HIV-seropositive individual with PBMCs from seronegative blood donors as recipient cells for the virus. Cocultures were incubated for 24 hr in medium containing phytohemagglutinin (PHA-P at 2  $\mu$ g/ml; Sigma, St. Louis, MO) and thereafter transferred to medium containing 5% interleukin 2 (Cellular Products, Buffalo, NY). Nine days postinfection 1 ml of virus-containing supernatant, as measured by p24 antigen capture assay, was added to PHA-P-stimulated PBMCs of another seronegative Swiss blood donor as recipient cells for the fresh viral isolate. After a 2-hr incubation with the virus cells were washed and divided into three cultures of equal size and treated with the oligodeoxynucleotides and transferred to the wells of a 48-well plate. One culture received no treatment; the second culture was treated with TFO A at 1  $\mu$ M concentration. After 24 hr supernatant was removed and fresh medium added containing the ODN at the same concentration (second spike). The third culture received treatment with TFO SC (random sequence control ODN) according to the same treatment schedule. (A) Antiviral effect of TFO A on HIV isolate obtained from a seropositive asymptomatic individual of category A1 of HIV infection. (B) Antiviral effect of TFO A on HIV isolate obtained from a seropositive symptomatic individual of category B of HIV infection. (C) Antiviral effect of TFO A on HIV isolate obtained from a seropositive symptomatic individual of category C3 of HIV infection.

tained from an asymptomatic individual of category A1 (compare scales of Fig. 3A and B). Such differences in the replicative capacity of two natural viral isolates may occur since these isolates were obtained from different individuals and different stages of the disease.

The third blood sample was obtained from a symptomatic seropositive individual of category C3 of HIV infection. Virus was isolated and cells were infected with TFO A and TFO SC as described above. HIV was not able to replicate in the culture that was treated with TFO A, no viral p24 antigen was detected, and the cells did not exhibit a cytopathic effect (Fig. 3C). In the control cultures p24 antigen expression starts 6 to 9 days postinfection. This viral isolate led to a readily discernible cytopathic effect.

#### *Synthesis of viral DNA in HIV-infected cells is prevented by TFO A treatment*

To test whether the treatment of HIV-infected PBMCs with TFO A prevented the synthesis of viral DNA, DNA on infected cells was extracted and subjected to PCR analysis of day 9 postinfection. To rule out false-negative results with the clinical isolate due to sequence variation, two pairs of primers were used, which allowed amplification of the R-U5-region of the viral LTR and part of the *gag* gene. The results shown in Table 1 demonstrate that addition of TFO A prevents synthesis of viral DNA in PBMCs infected with an HIV isolate of either a symptomatic individual (C3) or the laboratory strain of HIV-1, while both control cultures allow detection of viral DNA.

compared to the untreated culture. Both control cultures exhibited a cytopathic effect.

In a second experiment the inhibition of HIV-1 replication was tested with a fresh isolate obtained from a symptomatic seropositive individual of category B of HIV infection, using the same method of virus isolation and two consecutive treatments with TFO A and TFO SC as described above. Again, the presence of TFO A resulted in complete inhibition of viral replication as measured by p24 production (Fig. 3B). No syncytium formation was detectable in this culture. In the control cultures treated with the scrambled derivative TFO SC or no oligodeoxynucleotide, a strong p24 production starts 12 days postinfection and peaks at day 17 postinfection. Syncytium formation was observed in the two control cultures beginning at day 6 postinfection. The maximum levels of p24 produced with the viral isolate from a symptomatic individual of category B of HIV infection, are five times higher than those ob-

TABLE 1. DETECTION OF HIV VIRAL DNA BY POLYMERASE CHAIN REACTION

Sample	PCR results 9 days postinfection <sup>a</sup>	
	LTR	gag
1. PCR experiment <sup>b,c</sup>		
Without TFO	1.475	1.478
+TFO SC	1.474	1.481
+TFO A	0.017	0.012
HIV-negative DNA control	0.017	0.012
HIV-positive DNA <sup>d</sup> control	0.955	1.430
2. PCR experiment <sup>b,e</sup>		
Without TFO	≥2.50	≥2.50
+TFO SC	≥2.50	2.386
+TFO A	0.026	0.017
HIV-negative DNA control	0.024	0.018
HIV-positive DNA <sup>d</sup> control	1.419	2.484

<sup>a</sup>The mean values of duplicate experiments are shown.

<sup>b</sup>Cutoff values (mean  $A_{450} + 3$  SD) for positivity are as follows:

Experiment 1: LTR = 0.022 ( $n = 16$ ), gag = 0.015 ( $n = 16$ )

Experiment 2: LTR = 0.035 ( $n = 14$ ), gag = 0.024 ( $n = 14$ )

<sup>c</sup>The DNA of PBMCs infected with a clinical isolate of an individual of category C3 of HIV infection (shown in Fig. 3C) and cultured in the presence or absence of TFOs was subjected to PCR analysis.

<sup>d</sup>Approximately 5 dsDNA copies of HIV-1. A CEM-derived lymphocytic cell line infected with HIV-1 was used.

<sup>e</sup>The DNA of C81-66/45 T cells acutely infected with HIV-1 strain (HTLV-III<sub>B</sub>) and cultured in the presence or absence of TFOs was subjected to PCR analysis.

#### Evaluation of cytotoxicity of triplex-forming oligonucleotide treatment

To evaluate any cytotoxicity exerted by the ODN treatment, cell number and viability of the PBMC cells were determined on day 14 postinfection with the viral isolate of the symptomatic individual of category C3 of HIV infection and in two uninfected PBMC control cultures from the same seronegative blood donor. The viability of the cells of the culture treated with TFO A, in which viral replication was inhibited, amounted to 67%. As control for a possible cytotoxic effect of TFO a culture of uninfected PBMCs was incubated for 6 days in medium containing 1  $\mu$ M TFO A. The cells exhibited a viability of 68%, which is nearly identical to the viability of uninfected cells grown without TFO A (69%). The viability of the cells without treatment was reduced by the cytopathic effect of the virus to 28%. In the culture treated with the control TFO SC the cells exhibited a viability of 31%.

#### Analysis of the stability of triplex-forming oligodeoxynucleotides

To evaluate the contribution of the stability of TFOs to the efficacy of these compounds, TFO A and TFO SC were radioactively labeled and added to cell cultures (Fig. 4). The analysis of the remaining full-length ODNs after various times of incubation demonstrates a rather low stability, which is even more pronounced for TFO A than for TFO SC. This result indicated that TFO A is a potent inhibitor of viral replication.

## DISCUSSION

The PPT was selected as a viral target sequence for anti-retroviral therapy because it specifies an essential function in the retroviral life cycle as primer for plus-strand DNA synthesis.<sup>14,15</sup> The PPT can be used as target sequence for antisense

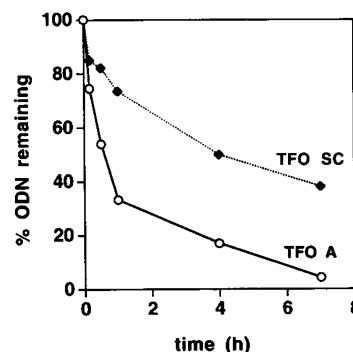


FIG. 4. Stability of TFO A and TFO SC. C81-66/45 cells were grown in the presence of radioactively labeled TFO A or TFO SC in RPMI medium containing 20% fetal bovine serum. Samples taken at the times indicated were fractionated by electrophoresis on a 12% denaturing polyacrylamide gel. The fraction of undegraded versus total TFO was quantitated by phosphorimaging and is indicated by percentage as a function of time (hours).

oligodeoxynucleotides.<sup>2</sup> The PPT consists of a purine-rich sequence. As shown for a site within the 5' end of the human *c-myc* gene,<sup>16</sup> such sequences are able to serve as target for triple-helix formation. Here we compared the inhibitory activity of 16-mer ODNs allowing Watson-Crick base pairing in the PPT region with the antiretroviral activity of TFO A. Only the phosphorothioate-modified antisense ODN exhibited a delaying effect on HIV replication, which, however, appears to be unspecific, since a similar effect was exerted by the randomized control oligodeoxynucleotide. The result therefore suggests that short phosphorothioate ODNs may have a sequence-independent effect that delays, but does not effectively inhibit, viral replication. This effect might reflect the results of Ojwang and co-workers,<sup>17</sup> demonstrating that phosphorothioated 18-mer ODNs exhibited RT inhibition independent of the sequence. We described earlier that a longer molecule, a 25-mer antisense ODN, allowing solely Watson-Crick base pairing with the extended PPT region, could exert a delaying effect on viral replication lasting from 4 to 6 days.<sup>1</sup> However, in contrast to the experiment described here, the control ODN exhibited no effect. This result suggests that with antisense ODNs directed against the PPT the specificity can be raised by increasing the length of the molecule. In contrast to the rather weak antiviral effects of 16-mer and 25-mer antisense ODNs, the inhibitory effect of TFO A was much more pronounced.

We therefore concentrated our efforts on the most effective inhibitor TFO A. When several different concentrations of TFO A were tested the concentration for inhibition of HIV ranged from 1 to 3  $\mu\text{M}$ . We have reported earlier that *in vitro* a triple helix as well as an enzyme inhibition were observed if TFO A was added to an RNase H cleavage reaction at concentrations higher than 1  $\mu\text{M}$ .<sup>1</sup> Inhibition of fresh patient-derived HIV isolates were performed at concentrations of 1  $\mu\text{M}$ . Previously the T cell line C81-66/45 was used as a target for an acute HIV infection with the laboratory strain HTLV-III<sub>B</sub> and tests for the efficacy of TFOs. On the basis of this assay, a test was designed that would allow us to test the inhibitory effect of TFO A in an acute infection with fresh field isolates of HIV. Virus was isolated from blood samples of three seropositive individuals. The amounts of viral p24 core antigen released into the culture supernatants, which reflect the amount of virus released, differed considerably between the cultures of the three isolates. Whether this is due to the wide spectrum of biological variability in viral replication rates that have been described in primary HIV isolates,<sup>18,19</sup> is not clear. Data on the high dynamics of HIV replication *in vivo*<sup>20</sup> indicate that variations of replication rates observed *in vitro* may reflect not only the replicative capacity of a viral isolate but also differences of the target cell populations used for isolation *in vitro*.<sup>21</sup> Peak levels of retroviral p24 antigen found in supernatants between 9 and 15 days postinfection ranged from 0.2 ng/ml in an untreated culture from a patient of category C3 up to 4.2 ng/ml in an untreated culture from another patient of category B.

Oligodeoxynucleotides, especially if thioated, can interfere with virus uptake, as has been described.<sup>22</sup> Oligodeoxynucleotides were therefore added to the cells 2 hr after the virus to avoid interference with binding of HIV to the CD4 receptor molecule. A control culture was kept without TFO to prove that under the conditions used, the amount of virus was sufficient to establish an infection. TFO SC, having the same base composition and identical modifications, showed no in-

hibitory effect on viral replication. Since TFO A is able to suppress viral replication during the whole culture period without further addition of the ODN, it can be concluded that the TFO A has an intracellular effect, e.g., by prevention of the establishment of infection. A third TFO molecule, consisting of a 54-nucleotide sequence and termed TFO B, was used as an additional specificity control. This molecule contains a GT-alternating sequence in its Hoogsteen base-pairing part while its Watson-Crick base-pairing part is unchanged. Thus TFO B cannot form a triple helix. TFO B exhibited no antiretroviral effect in cell culture (data not shown). Thus we conclude that the effect of TFO A in cell culture is caused specifically by the structure of this compound.

TFO A can combine with the viral target RNA to form a triple helix as shown in Fig. 5A and described previously by melting studies *in vitro*.<sup>1</sup> With the two exceptions, the TTA and TCG triplets in positions 17 and 18 of the extended PPT, the triple helix could be rather stable. Theoretically it could form with the incoming viral RNA as well as with progeny RNA.

However, the strength of the effect in cell culture and the lack of viral breakthrough during extended culturing suggest that no DNA provirus is formed either by triplex formation on the incoming viral RNA or by direct inhibition of the RT. On the basis of the nucleotide sequence the TFO A can form a hairpin-like

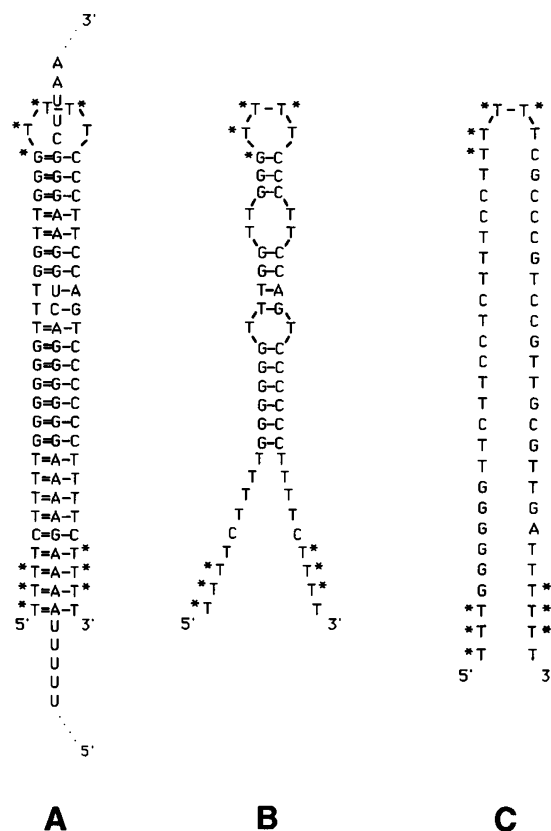


FIG. 5. Predicted secondary structures: (A) TFO A alignment on the target RNA sequence; =, Hoogsteen base pairing; -, Watson-Crick base pairing. Modified nucleotides are marked by asterisks. (B) Folding of TFO A was done by the StemLoop program of the Wisconsin Sequence Analysis Package. (C) Scrambled TFO sequence listed for comparison.



stem-loop structure shown in Fig. 5B. This structure of the TFO A resembles the DNA inhibitor molecules of the reverse transcriptase of HIV-1 described by Schneider and co-workers.<sup>23</sup> Some of these molecules evolved by the systematic evolution of ligands by exponential enrichment (SELEX) method possess different conserved helices interrupted by loops, bulges, or mismatched base pairs. These structural features are shared by TFO A. *In vitro* an inhibitory effect on the RT as well as on the RNase H has been shown to occur in the absence of a specific target RNA strand of TFO A.<sup>1</sup> Altogether the inhibitory effect of TFO A in cell culture may therefore be due to a superposition of a triple-helix effect and a direct enzyme inhibition. These dual inhibitory functions should be less likely to induce resistance, since two target sites are affected simultaneously.

Finally, it has been found that biologic effects, thought to be caused by the specific interaction of ODNs with their nucleic acid target, could be due to the presence of G tetrads and phosphorothioate modifications.<sup>24</sup> In our case this seems less likely, since TFO SC also contains five contiguous G residues and bears phosphorothioate modifications at the same positions as TFO A, while not being able to inhibit HIV infection. Apart from the potential use of the here-described compound for inhibition of HIV replication, it may contribute to a more fundamental understanding of nucleic acid-based therapies.

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